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# EXPRESSION OF P53 AND ISOFORMS IN BENIGN AND MALIGNANT LESIONS OF THE HEAD AND NECK

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**Short title:** Distribution of P53 and isoforms in benign and malignant lesions of head-neck regions.

**Key words:** P53, P53 isoforms, immunohistochemistry, benign and malignant squamous lesions, head and neck carcinogenesis.

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## ABSTRACT

**Background.** P53, a crucial suppressor of tumor formation, generates multiple isoforms, whose role in disease is still being defined.

**Methods.** By immunohistochemistry, we studied the expression of P53 protein and relative isoforms in benign papillomas (PA, n=9), inverted papilloma (IPA, n=10) and squamous cell carcinomas (SCC, n=21).

**Results.** In all lesions, P53 isoforms were significantly more expressed than P53. Immunoexpression of P53 matched with P53 isoforms in IPA as well as in SCC. Simultaneous immune-expression of P53 and related isoforms was double in SCC compared to IPA (10% vs 24%), while expression of P53 isoforms was strongly reduced (70% vs 43%). IPA showed the highest percentage of both reactive cases and immunostained cells expressing P53 isoforms.

**Conclusions.** We found the higher expression of P53 isoforms in IPA and SCC compared to PA, suggesting their role in local aggressiveness and malignant proliferation in head-neck lesions.

## INTRODUCTION

Tumor Protein (TP) 53 is a transcription factor controlling cellular proliferation and apoptosis by regulation of genes involved in cell-cycle arrest, DNA repair, and apoptosis (Moll et al., 2005; Vousden and Lane, 2007). By alternative initiation of translation and splicing mechanisms, TP53 may generate twelve varieties of transcripts such as FLP53, P53 $\beta$ , P53 $\gamma$ ,  $\Delta$ 40 $\alpha$ ,  $\Delta$ 40 $\beta$ ,  $\Delta$ 40 $\gamma$ ,  $\Delta$ 133 $\alpha$ ,  $\Delta$ 133 $\beta$ ,  $\Delta$ 133 $\gamma$ ,  $\Delta$ 160 $\alpha$ ,  $\Delta$ 160 $\beta$  and  $\Delta$ 160 $\gamma$ . (Bourdon et al., 2005, Braithwaite and Prives, 2006; Lane and Levine, 2010, Sourget et al., 2014). Alternative initiation of P53 transcription involves an internal promoter, namely P2, located in exon 4, while the splicing processes of P53 occur in intron 9 (Tuks and Crawford, 1989; Khoury and Bourdon, 2010; Marcel et al., 2010; Marcel et al., 2012.).

Canonical full length P53 (FLP3) protein is made of 393 amino acids (aa) building five major functional domains. These can be distinguished in amino terminal domain, showing two transactivation domains (TAD1 aa 1–40 and TAD2 aa 41–62), a proline-rich site (PXXP aa 63–94), a DNA-binding domain (DBD aa 95–290), a nuclear localization signal domain (NLS aa 291–325) and a carboxy-terminal oligomerization domain including oligomerization (OD aa 326–357) and negative-regulation sites (Neg aa 360–393) (Flaman et al., 1996; Levine et al., 2011). Therefore, the full set of P53 isoforms contains aa 160 through 325, retains NLS integrally, and DBD partially, and finally, may pass from cytoplasm to the nucleus binding specific DNA sequences (Courtois et al., 2002). Depending on tissue type, P53 isoforms act through the modulation of p53 transcriptional activity and by triggering target genes involved in cellular proliferation and apoptosis (Aoubala et al., 2011; Khoury and Bourdon, 2011).

Expressions of P53 isoforms have been reported in different malignant cancerous lesions such as breast cancer, ovarian, renal and colon carcinomas, suggesting a role of P53 isoforms in malignant proliferation (Bourdon et al., 2005; Marabese et al., 2008; Song et al., 2009; Fujita et al., 2009).

However, the role of P53 isoforms has not yet been clarified since even inflammatory lesions such as gastritis associated with *Helicobacter pylori* infection and benign colon adenoma may express spliced  $\alpha$  forms (Fujita et al., 2009; Wei et al., 2012). More recently, we have reported on different patterns of immunohistochemical expression of P53 isoforms also in benign thyroid lesions, suggesting for the first time their role in the development of such lesions (Trovato et al., 2016).

In the western world, squamous cell carcinoma of the head-neck region (SCCHN) has an incidence of nearly 5% (Jemal et al., 2007). Various studies proposed that a multi-step process, with accumulation of multiple genetic and epigenetic alterations, precedes the appearance of SCCHN (Mao et al., 2004). Genetic alterations occurring in pre-malignant lesions have been correlated with the development of locally aggressive phenotype (Wittekindt et al., 2012). In the context of multistep carcinogenesis of SCCHN, inverted papilloma (IPA) has a central role since it is a benign lesion in nature, but often displays an aggressive behavior in terms of local invasiveness, recurrence, and malignant transformation (Lawson et al., 2003). IPA lesions account for approximately 0.5% to 4% of all primary nasal tumors, and occur prominently in males (3:1), mainly in Caucasian populations (Sauter et al., 2007). From 2 to 27% of IPAs have been reported to be associated with malignant changes (Barnes, 2002; Von Buchwald, 2007).

Mutations of TP53 gene have been demonstrated by a frequency of 60–80%, and genetic alterations located in P53 core region, controlling DNA-binding, have been proposed as candidate markers of prognostic significance in SCCHN (Poeta et al., 2007). To date, expressions of p53, p53 $\beta$ , p53 $\gamma$ ,  $\Delta$ 133p53,  $\Delta$ 133p53 $\beta$  and  $\Delta$ 133p53 $\gamma$  have been reported in SCCHN, but IPA and benign papilloma (PA) have not been evaluated yet (Boldrup et al., 2007). Canonical P53 has been evaluated in IPA and SCCHN, but no data were available on P53 isoforms (Oncel et al., 2011).

The aim of our study was to investigate the expression of P53 paired with P53 isoforms in PA, IPA and SCCHN to determine what role P53 and related isoforms may have in these malignancies and in multistep carcinogenesis of the head-neck region.

## MATERIALS AND METHODS

### **Patients and Tissue Collection**

For this study, 40 unrelated patients were recruited, 37 men and 3 women (mean age  $\pm$  SD: 68  $\pm$  18), of which 9 were affected by benign lesions and 31 by malignant proliferations of the head-neck region. Each patient was seen at the Unit of Odontostomatology of the University of Messina, where they received careful medical evaluation, including recording of past medical history and physical examination. Clinical diagnosis was supported by computed tomography analysis, and the benign lesions were catalogued as benign papillomas (PA, n=9), while the malignant lesions were HPV-unrelated tumors and were grouped into 10 inverted papillomas (IPA) and 21 carcinomas (SSCHN). None of the patients had received chemotherapeutic drugs before surgery.

### **Methods and immunohistochemical evaluation**

Each surgical specimen was evaluated at the Unit of Integrated Ultrastructural Pathology, Department of Human Pathology, University of Messina. Specimens were fixed in 4% neutral buffered formalin and embedded in paraffin. Each block was cut into serial sections of 5-mm thick to carry out histochemical staining, such as hematoxylin and eosin (H&E), Periodic acid Schiff (PAS), and immunohistochemistry (IHC). According to the World Health Organization (WHO) standard criteria for tumor classification, the 21 carcinomas were catalogued as squamous cell carcinomas (SCCHN), and graded as well-differentiated (n=2), moderately differentiated (n =10) and poorly differentiated (n=9) (Barnes, 2002). In each section used for immunohistochemical procedures apparently normal peritumoral tissue was also included.

IHC was carried out on 5- $\mu$ m thick sections of selected blocks including either lesion or peri-lesion normal tissue. Two distinct monoclonal mouse antibodies were used to detect P53 and its isoforms.

The former recognizes an epitope located in P53 TAD1 region, between amino acids 20–25, and reacts with FLp53, P53 $\beta$  and  $\gamma$  (DO-7, 1:100; DAKO, Carpinteria, CA, USA); the latter antibody recognizes an epitope located within the P53 DBD region, between amino acids 181–190, and binds the full set of P53 isoforms (DO-11, 1:500; Novus Biologicals Europe, Cambridge, UK; [www.novusbio.com/NB100-65557](http://www.novusbio.com/NB100-65557)).

The antigen retrieval technique as described by Gown et al. (Gown et al., 1993) was used. The sections were deparaffinized with xylene and rehydrated through graded alcohol. Endogenous biotin was inactivated by addition of 0.05% (v/v) solution of streptavidin in phosphate-buffered saline (PBS) and endogenous peroxidase activity was suppressed by adding 0.3% (v/v) solution of 3% (v/v) H<sub>2</sub>O<sub>2</sub> in absolute methanol. Slides, placed in 10 mM citrate buffer (pH 6.0), were heated for 15 min in a microwave oven (Whirlpool AVM 300, power set at 500 watts) for three equal time periods. IHC procedures were performed by using an automated slide processing system designed by Autostainer Link 48 system (Dako). IHH staining was visualized by the LSAB system (Universal Dako LSAB® + Kit, Peroxidase, Carpinteria, CA, USA) whereas the colour reaction was developed by 3,3'-diaminobenzidine (DAB, Sigma) activated with 0.05% hydrogen peroxide. Sections were counterstained with Mayer's haematoxylin, dehydrated and mounted. Specificity of the binding was assessed on appropriate negative controls, either by omitting the primary antiserum or by replacing the primary antiserum with normal mouse serum. In each of these conditions, no staining was evident. An immunoabsorption test was performed to confirm the specific immunoreactivity of each MAb. Specimens of breast carcinoma were used as appropriate positive controls for P53 antibodies.

P53 and P53 isoforms immunoreactions were estimated using the following criteria: (i) number of positive cases; (ii) number of reactive epithelial cells per case: the count of reactive cells was based on evaluation of 1000 cells/case, using 40 X magnification; (iii) sub-cellular location of the



staining: cytoplasm and nucleus; (iv) semiquantitative grading of staining using a “Quickscore” method as proposed by Detre et al. (Detre et al., 1993). This method was based either on the proportions of immunostained cells or on the intensity of labelling. The immunostained cells were scored on a system from I to IV (I = 0-4% of cells; II = 5-19%; III = 20-39%; IV = 40-59%). The intensity of immunostaining was graded from 0 to 3, corresponding with absence of labelling, weak, intermediate, and strong immunostaining, respectively. Grade I of immunoreactions was considered as unreactive; grade II of stained cells plus grade 2 or 3 of stain was indicated as class A; grade III plus grade 2 or 3 was recorded as class B; grade IV plus grade 2 or 3 was indicated as class C. Histological and immunohistochemical evaluations were carried out blindly by three different pathologists (M.T., M.S. and V.C.) with an inter-observer concordance of nearly 100%. Minimal inter-observer discrepancies were overcome by mean value.

### **Statistical Analysis**

Data were evaluated for normal distribution and variance (mean  $\pm$  standard deviation) and analyzed by the two-tailed Student's t-test and  $\chi^2$  test with Yates' correction for continuity. Level of statistical significance was set at  $P < 0.05$ .

## RESULTS

### Histological evaluation

Microscopic diagnostic signs in PA cases included columnar and squamous epithelium cells, microcysts, mucinous cells associated with rare neutrophils, and microabscesses with reactive epithelial changes and oncocytic cells.

Histological features of IPA were compatible with a benign proliferation composed of well differentiated columnar and ciliated respiratory epithelium focally showing squamous differentiation, along with invagination of the epithelium into underlying stroma. Necrosis was usually absent.

Common microscopic appearance of SCCHN was seen in the areas of keratinizing, infiltrative nests of tumoral cells and surrounding peritumoral stromal desmoplasia. Squamous maturation was recognized by features of keratinization. Malignant cells were polygonal with distinct cell borders and abundant, eosinophilic cytoplasm. Necrosis foci were frequent. IPA signs were not detected.

### Immunohistochemistry

Immunohistochemistry results are summarized in **Table 1** and illustrated in **Figures 1 and 2**. All reactive samples showed immunostaining for FLP53 and the full set of P53 isoforms in both the cytoplasmatic and the nuclear compartments. P53 isoforms were more frequently immune-expressed than the canonical P53 both in benign and malignant lesions (5/9 vs 0/9 and 16/31 vs 6/31;  $P < 0.05$ ) (**Table 1**). With the exceptions of PA and well differentiated SCCHN, which did not show P53 immunostaining, canonical P53 expression matched P53 isoform immunoreactions.

Simultaneous immunoexpression of P53 and related isoforms was more than double in SCCHN compared to IPA (10% vs 24%;  $P=0.66$ ), even if such differences didn't reach statistical significance, probably due to the small numbers; in sharp contrast, expression of the P53 isoforms was greatly reduced ( $P=0.022$ ). (**Table 1**).

Immunoreactivity of P53 isoforms was quite similar in benign and malignant lesions, appearing in approximately half of cases. However, when evaluating the cellular distribution of immunostaining, a different pattern of P53 isoform immunoreactions emerged. Only malignant lesions showed class C immunostaining and had the highest proportion of immunoreactive cells for P53 isoforms.

P53 isoforms were more highly expressed in IPA lesions compared to PA (70% vs 55%) and SCCHN (70% vs 43%) (Table2). Furthermore, the IPA lesions showed the widest proportion of immunostained epithelial cells. While no lesion fell into the A category, all the IPA lesions belonged to both class B and C. Peritumoral normal tissue was unstained at all.

From SCCHN grading, it emerged that P53 isoforms were mainly expressed in moderate and poorly differentiated variants, which also showed the highest proportion of immunostained squamous carcinomatous cells. Furthermore, in reactive SCCHN, grade A immunostaining for P53 isoforms was observed in the adjacent peritumoral normal tissue.

## DISCUSSION

In the present study, we evaluated the expression of P53 and its isoforms in a series of head-neck lesions encompassing the whole spectrum of epithelial squamous proliferation, from benign lesions (PA) along with local aggressive malignancy (IPA) to true malignancy (SCCHN).

In our study we first evaluated the expression of P53 and its isoforms in three types of head-neck lesions, ranging from benign papillomas to malignant SSCHN passing through IPA, in comparison with the peri-tumoral normal tissue in order to identify differences in P53 and isoforms expression between such lesions. We firstly demonstrated that P53 isoforms are expressed in all the steps of squamous carcinogenesis, even in the early stages, by a percentage of cases close to 50%, while P53 expression seems to be associated with local aggressiveness appearance and true malignancy.

Secondly, our study documented a distribution of P53 isoforms significantly different in IPA compared to PA and SCCHN. In fact, IPA showed the highest percentage of reactive cases and the largest number of immunoreactive epithelial cells, suggesting a role of P53 isoforms in the development of local aggressiveness. Indeed, such a role may be hypothesized, since the  $\Delta 133$  isoforms of P53 have been reported as fundamental to stimulate migration and tubulogenesis processes in tumour cells (Bernard et al., 2013). Taken all of the above, P53 isoforms might act in IPA lesions by driving local invasion of epithelial cells.

The third interesting point of our results was the demonstration that simultaneous immunoreactions of P53 and its isoforms were increased in SCCHN compared to IPA, while, conversely, expression of the P53 isoforms was reduced in SCCHN. In fact, we found that 60% of IPA displayed the expression of P53 isoforms only, compared to 19% of SCCHN.

To date, very few reports have investigated the role of P53 isoforms in head-neck lesions, although it is currently accepted that disruption of the P53 pathway is one of the most common genetic alteration in head-neck cancers. Mutations of P53 have been reported in up to 80% of SSC (Poeta et al., 2007), and canonical p53 over-expression was been found to be associated with malignant potential in pre-malignant head-neck lesions (Oncel et al., 2011; Gujrathi et al., 2003). Gujrathi et al. found that four of the five cases of malignancy associated with IPA overexpressed p53, while none of the benign cases of IPA demonstrated such an over-expression. More recently, Oncel S. et al. studied the expression of the cell cycle regulators P53, P62, P21 and P27 in IPA and SCC, revealing elevated levels of P53 expression in SCC compared to IPA. On this basis, it has been suggested that overexpression of p53 may serve as a marker of malignant transformation of IPA, to screen out papilloma lesions with a potential for dysplasia or carcinoma. However, only the canonical P53 expression was tested, and benign PA lesions, as well as the peritumoral normal tissue, were not evaluated in this study.

Prior to us, Boldrup and co-workers first reported the expression of canonical P53 and numerous P53 isoforms, P53 $\beta$ , P53 $\gamma$ ,  $\Delta$ 133 P53,  $\Delta$ 133 P53 $\beta$  and  $\Delta$ 133 P53 $\gamma$ , both in SCCHN lesions and in paired normal tissues (Boldrup et al., 2007). In this study, SCC lesions were studied paired with normal tissues, but benign papillomas, as well as IPA, were not evaluated. Our report on the distribution of P53 isoforms in different steps of squamous carcinogenesis may add novel findings to the debate on the relationship between such lesions and molecular pathways regulating cell cycle and apoptosis. Taken together, our data suggest that analysis of P53 along with isoforms expression may be a suitable candidate marker to explore squamous cell proliferation referring to invasive malignancy.

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## LEGEND FOR FIGURES

**Figure 1.** Immunoreactions for P53 isoforms in papilloma, inverted papilloma, squamous cell carcinoma and peritumoral normal tissue of squamous cell carcinoma.

Panel A and D: class B immunoreactivity in a papilloma and normal tissue surrounding squamous cell carcinoma, respectively; that is, 20-39% of stained cells plus intermediate or strong immunostaining as specified under Material and Methods (original magnification X200).

Panels B and C: class C immunostaining in an inverted papilloma and poorly differentiated squamous cell carcinoma, respectively; that is, 40-59% of stained cells plus intermediate or strong immunostaining (original magnification x400).

**Figure 2.** Immunoreactions for P53 in cases of papilloma, inverted papilloma, squamous cell carcinoma and paired peritumoral normal tissue.

Panel A and D: unstained P53 papilloma and normal tissue surrounding poorly differentiated squamous cell carcinoma, respectively (original magnification x200).

Panels B: class A immunostaining in an inverted papilloma; that is 5-19% of stained cells plus intermediate or strong immunostaining (original magnification x400).

Panel C: class B immunoreaction in moderate differentiated squamous cell carcinoma (original magnification x400).

**Table 1.** Immunoreactivity for P53 and its isoforms in our series of 40 patients affected by either benign or malignant lesions of head and neck.

Lesions	Number of cases	P53				P53 isoforms			
		Immunoreactive cases (%)	Cellular distribution of immunoreactions (%)			Immunoreactive cases (%)	Cellular distribution of immunoreactions (%)		
		Reactive	A*	B**	C***	Reactive	A*	B**	C***
<b>Benign</b>	<b>9</b>	<b>0</b>	<b>0</b>	<b>0</b>	<b>0</b>	<b>5 (55%)</b>	<b>40</b>	<b>60</b>	<b>0</b>
<b>Malignant</b> ( <i>Inverted papilloma and Squamous carcinoma</i> )	<b>31</b>	<b>6 (19%)</b>	<b>50</b>	<b>50</b>	<b>0</b>	<b>16 (52%)</b>	<b>12</b>	<b>38</b>	<b>50</b>
<b>Pathological lesions</b>									
<b>Papilloma</b>	<b>9</b>	<b>0</b>	<b>0</b>	<b>0</b>	<b>0</b>	<b>5(55%)</b>	<b>40</b>	<b>60</b>	<b>0</b>
<b>Inverted papilloma</b>	<b>10</b>	<b>1 (10%)</b>	<b>100</b>	<b>0</b>	<b>0</b>	<b>7 (70%)</b>	<b>0</b>	<b>29</b>	<b>71</b>
<b>Squamous carcinoma</b>	<b>21</b>	<b>5 (24%)</b>	<b>40</b>	<b>60</b>	<b>0</b>	<b>9 (43%)</b>	<b>22</b>	<b>12</b>	<b>66</b>
<b>Squamous carcinoma</b> Histological grading									
Well differentiated	<b>2</b>	<b>0</b>	<b>0</b>	<b>0</b>	<b>0</b>	<b>0</b>	<b>0</b>	<b>0</b>	<b>0</b>
Moderately differentiated	<b>10</b>	<b>2 (20%)</b>	<b>100</b>	<b>0</b>	<b>0</b>	<b>5 (50%)</b>	<b>40</b>	<b>0</b>	<b>60</b>
Poorly differentiated	<b>9</b>	<b>3 (33%)</b>	<b>0</b>	<b>100</b>	<b>0</b>	<b>49 (44%)</b>	<b>0</b>	<b>25</b>	<b>75</b>

The immunoreactivities for  $\Delta 133$  isoforms of P53 were classified as Class A, B, C and D by using the “Quickscore” method, as reported in Material and Methods.



